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# ATP Bioluminescence Assay for Estimation of Microbial Populations of Fresh-Cut Melon<sup>†</sup>

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## ABSTRACT

Estimation of microbial numbers in foods by conventional microbiological techniques takes days, so there is a need for faster methods that can give results in minutes. Research was undertaken to investigate the use of bioluminescent ATP determination and a firefly luciferase assay to estimate the initial population of aerobic mesophilic bacteria on fresh-cut melons immediately after preparation and during storage at 5 or 15°C for up to 12 days. Populations of aerobic mesophilic bacteria on fresh-cut cantaloupe prepared immediately from unsanitized whole melons averaged 3.42 log CFU/g, corresponding to an ATP value of 5.40 log fg/g. Populations for fresh-cut honeydew prepared from unsanitized whole melon averaged 1.97 log CFU/g, corresponding to an ATP value of 3.94 log fg/g. Fresh-cut pieces prepared from cantaloupe or honeydew melons sanitized with either chlorine (200 ppm free chlorine) or hydrogen peroxide (2.5%) had similar ATP values: 3.1 log fg/g (corresponding to bacterial counts 1.7 log CFU/g) for cantaloupes and 2.6 log fg/g (corresponding to bacterial counts of 0.48 CFU/g) for fresh-cut honeydew. Positive linear correlations for ATP concentrations and microbial populations were found for fresh-cut cantaloupe ( $R^2 = 0.99$ ) and honeydew ( $R^2 = 0.95$ ) during storage at 5°C for up to 12 days. ATP values in fresh-cut melons inoculated with either aerobic mesophilic bacteria or yeast and mold were significantly higher ( $P < 0.05$ ) than control values and parallel total plate counts on plate count agar. Results of this study indicate that the bioluminescent ATP assay can be used to monitor total microbial populations on fresh-cut melon after preparation and during storage for quality control purposes to establish specific sell-by or consume-by dates.

Fruits and vegetables are frequently in contact with soil, insects, animals, and humans during growth and harvesting. Thus, by the time it reaches the packinghouse, most fresh produce retains microbial populations of 4 to 6 log CFU/g (3, 4). The surface of cantaloupe harbors greater populations of native microflora than does honeydew (18). Transfer of microorganism from melon surfaces to fresh-cut pieces has been reported, and the transfer rate was higher for cantaloupe than for honeydew (18). Prepared fresh-cut melon in the supermarket is becoming very popular with the U.S. consumer because of the benefits of a diet rich in fruits and vegetables. Retail fresh-cut melon is prepared in the supermarket or by regional distributors. National distribution is difficult because of quality and safety concerns. Therefore, knowledge of the microflora on whole and fresh-cut melons should help processors in implementing hazard analysis critical control point plans and good manufacturing practices. However, estimation of the microbial load on a particular foodstuff is problematic, especially when the surface of interest is uneven, as in the case of whole cantaloupe (20), and obtaining a representative sample for examination is often difficult (15).

Estimation of microbial numbers in foods using conventional microbiological techniques takes at least 2 to 3

days, and by that time foods are often already in the marketplace. Therefore, faster methods that can give results in minutes to hours are needed. Bioluminescent ATP techniques have been used to estimate the numbers of bacteria in various microbiological media, on food contact surfaces, and in foods (1, 2, 5–9, 11–13, 16, 17, 22). This technique for rapid measurement is based on the premise that all living things contain ATP and that intracellular ATP levels must be constant for cells to maintain normal physiological activities. Therefore, the concentration of ATP determined in a sample is proportional to the actual cell number (17, 21). Previously, we recommended a bioluminescence ATP assay for quick estimation of total microbial load on sanitized cantaloupe surfaces rather than the conventional microbial methods, which require incubation for days before results are obtained (20). In this study, we investigated the efficacy of a bioluminescence ATP assay in determining the microbiological quality of fresh-cut melon immediately after preparation and during refrigerated storage so that this information could be used to establish specific sell-by or consume-by dates.

## MATERIALS AND METHODS

**Cantaloupe and honeydew melons.** Unwaxed cantaloupes (1,622 ± 24 g, western shippers) and honeydew melons (1,856 ± 38 g) purchased from a local wholesale distribution center were stored at 4°C until used. Before use, melons were unpacked and placed on the laboratory bench for approximately 18 h to allow them to come to room temperature (approximately 23°C).

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† Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

**Isolation of native aerobic mesophilic bacteria and yeasts and molds from melon surfaces.** Random samples of 70 plugs per cantaloupe weighing approximately 25 g (with a total surface area of 266 cm<sup>2</sup>) were blended (commercial Waring Blender, Dynamic Corp, New Hartford, Conn.) at speed level 5 for 1 min with 75 ml of 0.1% peptone water. Decimal dilutions of the sample were made with 0.1% peptone water, and aliquots (0.1 ml) were plated in duplicate on agar media. Mesophilic aerobes were cultured on plate count agar (PCA; BBL, Becton Dickinson, Sparks, Md.) by incubation at 30°C for 3 days. Yeasts and molds were cultured using Czapek malt agar (CMA; Sigma, St. Louis, Mo.) (14). Yeast and mold colonies from the CMA plates were scraped into 5 ml of Sabouraud maltose broth (SMB; BBL, Becton Dickinson), mixed, and then incubated at 25°C for 4 days. Aerobic mesophilic bacteria isolated from PCA plates were suspended in 10 ml of brain heart infusion broth (BHIB) with a sterile transfer loop and incubated at 30°C for 3 days. A final transfer (0.2 ml) of each class of native microflora was made into 20 ml of BHIB, which was incubated at 30°C for 3 days, or into SMB (yeasts and molds only), which was incubated at 25°C for 4 days under static conditions (19). Bacterial cells and the yeasts and molds were harvested by centrifugation (10,000 × g for 5 min) at 4°C, and the cell pellets were washed in salt-peptone (0.85% NaCl, 0.05% Bacto-peptone; BBL, Becton Dickinson). The final cultures of native bacteria and yeasts and molds used to inoculate fresh-cut pieces were adjusted to contain final populations of 2, 3, 4, or 5 log CFU/ml.

**Washing treatments.** A commercial bleach containing 5.25% sodium hypochlorite (Clorox, Clorox Company, Oakland, Calif.) was diluted in sterile water to obtain a wash solution containing 200 ppm of free chlorine. The pH was adjusted to 6.4 ± 0.1 by adding citric acid (Sigma). Free chlorine in the solution was determined with a chlorine test kit (Hach Co., Ames, Iowa). A second wash solution was prepared from a 30% stock solution of hydrogen peroxide (Fisher Scientific, Suwanee, Ga.) that was diluted to 2.5% in sterile water. Washing treatments were performed by totally submerging the melons in 3 liters of sterile tap water or water containing 200 ppm chlorine or 2.5% hydrogen peroxide. Melons were manually rotated to assure complete coverage and contact of surfaces with the wash solution for 5 min. Washed melons were placed on crystallizing dishes inside a biosafety cabinet to dry for 1 h.

**Preparation and inoculation of fresh-cut pieces.** To prepare fresh-cut pieces, unwashed whole cantaloupe or honeydew melons and those washed with water or solutions of chlorine or hydrogen peroxide were each cut into four wedges using a sterile knife. The rinds were removed, and the flesh was cut into approximately 3-cm cubes. Fresh-cut pieces (approximately 970 g per melon) were divided into two groups of 450 g each. To determine the correlation between ATP concentration and microbial populations, pieces were submerged for 2 min in inocula containing either native aerobic bacteria or yeasts plus molds at 2, 3, 4, or 5 log CFU/ml. As a control, pieces were submerged in salt-peptone alone for 2 min. Inoculated and control fresh-cut pieces were placed for 1 h on a perforated dish inside a biosafety cabinet to drain any excessive fluid. For storage experiments, a 100-g sample of fresh-cut pieces inoculated with aerobic bacteria at 4 log CFU/ml was placed inside a 9.75-in. (24.77-cm) three-pocket Tub, Tall plastic bowl (Mach 2, Rock-Tenn Company, Franklin Park, Ill.) and stored at 5 or 15°C for up to 15 days. On day 0, samples were homogenized within 5 h of being prepared, and populations of aerobic bacteria and ATP concentration were determined.

**Microbiological analyses.** Approximately 100 g of melon flesh was placed in a Stomacher bag with 200 ml of 0.1% peptone water and pummeled for 30 s in a Stomacher (model 400, Dynatech Laboratories, Alexandria, Va.) at medium speed. Total aerobic bacteria were enumerated on PCA, and yeasts and molds were enumerated on CMA as previously described (14, 18, 19). At days 0 and 3 of storage of fresh-cut pieces, and undiluted (0.1 ml) portion of homogenate of fresh-cut melon was plated. For fresh-cut pieces stored for more than 3 days, decimal serial dilutions of the homogenates were plated (0.1 ml) on the agar media.

**Bioluminescence ATP assay.** ATP was extracted by boiling homogenate for 5 min in a Tris-EDTA solution (0.1 M Tris and 2 mM EDTA, pH 7.75) (20). Portions (1 ml) of homogenates prepared for microbiological analysis were mixed with 100 µl of apyrase (Sigma) and then incubated at 35°C for 1 h to destroy somatic ATP (21). Four milliliters of extraction solution was added to each sample, which was then mixing with a vortex mixer for 1 min, heated in boiling water for 5 min, and cooled to room temperature in an ice bath. The ATP concentration was determined by the luciferin-luciferase reaction, using an ATP bioluminescent assay kit (Turner Design, Sunnyvale, Calif.). Bioluminescence ATP assays were performed using a TD-20/20 (DL Ready) luminometer (Turner). The generated light signal was measured after a 3-s delay time and a 14-s integration time. Assays of standard amounts of purified ATP (Turner) were used to calculate ATP concentrations, which were expressed as log femtogram per gram of fresh-cut melon. Controls for background luminescence (50 µl of Tris-EDTA buffer) were run, and the readings were subtracted from readings for ATP concentration. Possible inhibition of the luciferase reaction by extracts or residues from the fresh-cut homogenates was corrected for by addition of known amounts of ATP standard to the reaction vial followed by addition of the luciferase enzyme.

**Data analysis.** All experiments were done in triplicate, and duplicate samples were analyzed at each sampling time. Data were subjected to analysis of variance using the Statistical Analysis System Program (SAS Institute, Cary, N.C.). The SAS program was used for correlation of ATP concentrations with the CFU. Significance of the differences ( $P < 0.05$ ) between mean values of number of cells and ATP concentrations was determined using the Bonferroni least significant difference method.

## RESULTS AND DISCUSSION

**ATP concentrations in fresh-cut pieces.** Previously, we compared use of chloroform-water solution, Tris-EDTA solutions, and Extralight solution for extracting ATP (20). Although chloroform-water gave the highest ATP concentration, followed by Tris-EDTA, we used the Tris-EDTA method for this study because of cost and worker safety considerations.

The concentrations of bacterial ATP extracted from the cantaloupe homogenates were higher than those for honeydew for both rind and fresh-cut pieces, reflecting a higher microbial load for the cantaloupe. The concentrations of ATP from the control cantaloupe rind homogenates averaged 9.23 log fg/cm<sup>2</sup>, which corresponds to a population of aerobic bacteria averaging 7.54 log CFU/cm<sup>2</sup>. For honeydew melon, ATP concentrations for the control rind homogenates averaged 4.53 log fg/cm<sup>2</sup>, which corresponds to a bacterial population of 3.54 log CFU/cm<sup>2</sup>. The ATP concentrations determined in homogenates of whole cantaloupe

TABLE 1. *Microbial populations of fresh-cut melons estimated by total aerobic plate counts and ATP bioluminescence assay<sup>a</sup>*

Treatment	Cantaloupe		Honeydew	
	Aerobes (log CFU/g)	ATP (log fg/g)	Aerobes (log CFU/g)	ATP (log fg/g)
Control	3.42 ± 0.14 A	5.40 ± 0.11 A	1.97 ± 0.11 A	3.94 ± 0.12 A
Water	3.19 ± 0.15 A	3.59 ± 0.12 B	1.41 ± 0.10 B	3.43 ± 0.12 B
Chlorine (200 ppm)	1.83 ± 0.13 B	3.12 ± 0.23 C	0.48 ± 0.14 C	2.64 ± 0.16 C
Hydrogen peroxide (2.5%)	1.61 ± 0.12 B	3.18 ± 0.24 C	0.48 ± 0.16 C	2.59 ± 0.13 C

<sup>a</sup> Values are means of duplicate samples from three trials. For each column, means not followed by the same letter are significantly different ( $P < 0.05$ ). Aerobic plate counts were determined immediately before ATP extraction. Tris-EDTA was used for all ATP extractions.

rind in this study were higher than those reported previously (20). These higher values can be attributed to the higher populations of bacteria on the cantaloupe melons in this study compared with the previous study. The higher populations of native microflora on the surfaces of whole and fresh-cut cantaloupe than on the surfaces of whole and fresh-cut honeydew is consistent with results of our previous study (18).

Total plate counts on PCA and ATP concentrations from fresh-cut pieces prepared from unsanitized or sanitized whole cantaloupes are shown in Table 1. Total plate counts on PCA and ATP concentrations from fresh-cut pieces prepared from sanitized whole cantaloupes were significantly lower ( $P < 0.05$ ) than the corresponding values for unsanitized melons. Fresh-cut honeydew pieces prepared from untreated whole honeydew melon had lower ATP concentrations and total plate counts than did fresh-cut cantaloupe pieces. Water washes of whole melons did not result in significant reductions ( $P > 0.05$ ) in total aerobic counts or ATP concentrations for the fresh-cut pieces. Total aerobic counts and ATP concentrations for fresh-cut pieces prepared from cantaloupes sanitized with chlorine or hydrogen peroxide were not significantly different ( $P > 0.05$ ).

There was a linear relationship ( $R^2 = 0.95$ ) between

numbers of aerobic bacteria and ATP concentrations for both uninoculated melon pieces and pieces inoculated with aerobic bacteria (data not shown). Similarly, a linear relationship ( $R^2 = 0.99$ ) between total aerobic counts and ATP concentrations was found for both uninoculated melon pieces and pieces inoculated with yeasts and molds (data not shown). The minimum detection limit for which accurate bacterial estimation was achieved was 3.0 log fg/g for fresh-cut cantaloupe and 2.6 log fg/g for fresh-cut honeydew. Below this threshold, ATP determinations were highly variable, in agreement with earlier studies (12, 13, 20).

**Use of ATP concentrations for estimation of microbial contamination of fresh-cut melon.** In this set of experiments, the relationship between ATP and the microbial populations on inoculated fresh-cut melon was investigated. Total bacterial populations in fresh-cut pieces prepared from sanitized whole honeydew averaged 0.48 log CFU/g with an ATP concentration of approximately 2.6 log fg/g, whereas total bacteria in fresh-cut cantaloupes prepared from sanitized whole cantaloupes was approximately 1.7 log CFU/g with an ATP concentration of approximately 3.10 log fg/g. Regardless of the sanitizing treatment used, surfaces of whole and fresh-cut cantaloupes always had

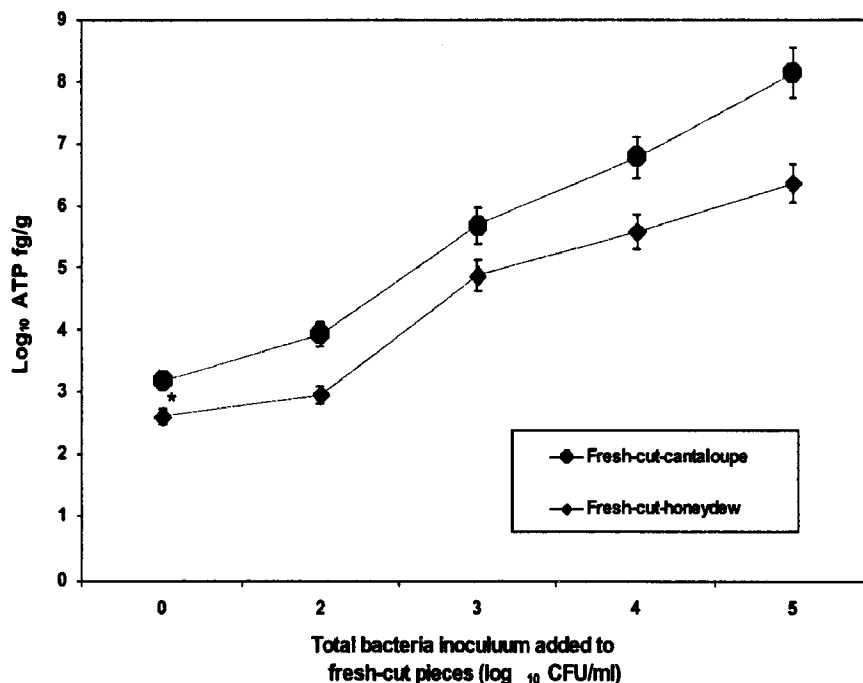
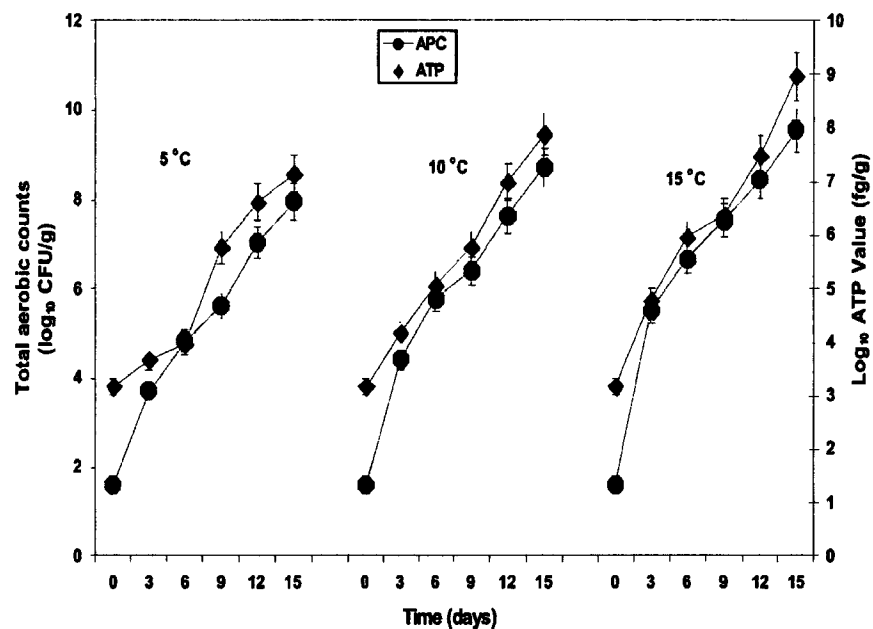


FIGURE 1. Relationship between ATP concentrations and total aerobic plate counts for fresh-cut cantaloupe artificially inoculated with aerobic mesophilic bacteria. Values are means ± standard deviation of three determinations. An asterisk indicates the initial bacterial count and the corresponding ATP concentration.

FIGURE 2. Total aerobic plate counts and corresponding ATP concentrations of fresh-cut cantaloupe following storage at 5, 10, and 15°C for up to 15 days. Whole melons were sanitized with chlorinated water. Values are means  $\pm$  standard deviation of three determinations.



higher microbial populations than did surfaces of honeydew melons, and initial ATP concentrations also were higher. When fresh-cut pieces from both melons were contaminated with aerobic bacteria at  $10^2$  to  $10^5$  CFU/ml, the ATP concentrations increased (Fig. 1). There was a linear relationship between ATP concentrations (log fg/g) and aerobic plate counts (log CFU/ml) for both fresh-cut cantaloupe and fresh-cut honeydew. However, both the ATP concentrations and the total bacterial counts in fresh-cut pieces of cantaloupe were significantly higher ( $P < 0.05$ ) than the corresponding values for fresh-cut pieces of honeydew. ATP concentrations in fresh-cut pieces inoculated with yeasts and molds were higher than concentrations in fresh-cut pieces inoculated with similar populations of aerobic mesophilic bacteria (data not shown). The higher ATP concentrations in fresh-cut melon pieces inoculated with yeasts

and molds may be due to the larger cell size and volume for yeasts compared with aerobic microbes.

Some researchers have reported possible interference and/or quenching of bioluminescence by the chemical extractant that may affect the reported ATP concentrations. Selan et al. (16) demonstrated the ability of chemical extractants to contribute to the quenching of bioluminescence. Velazquez and Feirtag (23) observed a transition from enhancement to quenching for chemical sanitizers such as alkaline foam, acid foam, commercial sodium hypochlorite, and D-limonene. They concluded that this effect was concentration dependent and that enhancement occurred at lower concentrations. Greig (8) reported that ATP estimation of cell numbers by bioluminescence may agree closely with colony counts on agar plates for gram-positive cocci (staphylococci and streptococci). For example, the ATP

FIGURE 3. Total aerobic plate counts and corresponding ATP concentrations of fresh-cut honeydew following storage at 5, 10, and 15°C for up to 15 days. Whole melons were sanitized with chlorinated water. Values are means  $\pm$  standard deviation of three determinations.

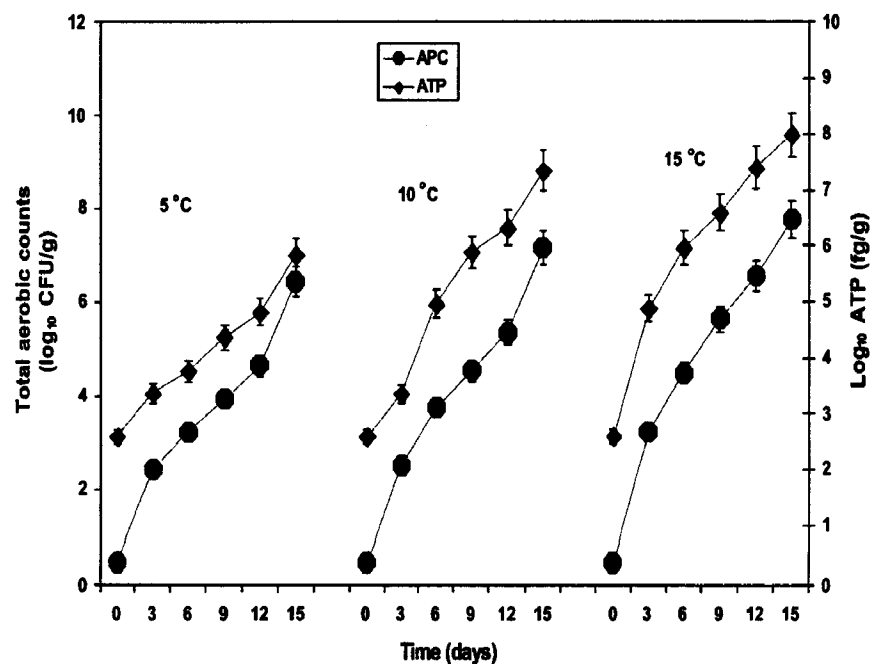


TABLE 2. Linear regression and correlation coefficients for correlation of total aerobic plate counts on PCA with ATP concentrations in fresh-cut cantaloupe and honeydew melons

Treatment	Pearson correlation coefficients <sup>a</sup>					
	Cantaloupe			Honeydew		
	a	b	r	a	b	r
Fresh-cut melon pieces prepared after purchase	1.491	0.923	0.999	2.603	0.648	0.998
Fresh-cut pieces from whole melon sanitized with 200 ppm Cl <sub>2</sub>	1.848	0.976	0.999	0.665	0.602	0.998

<sup>a</sup> Values represent the strength of the relationship between total aerobic plate count and ATP concentration, expressed as the ratio of SS (regression) to [SS (regression) + SS (within)], where *a* is an estimate of the y-intercept, *b* is an estimate of the slope, and *r* is the correlation coefficient.

concentration may predict a value of  $10^5$  CFU/ml, whereas the colony count is  $10^4$  CFU/ml or less. The reason for such a discrepancy might be that *Staphylococcus* and *Streptococcus* organisms grow in bunches and chains, respectively. Therefore, each CFU of these organisms may in fact represent 10 to 20 bacterial cells. This phenomenon may not apply to the present study because the blending process utilized should have broken up any bacterial clumps originating from the cantaloupe surfaces. Karl (10) reported that vacuum filtration can be used to increase the sensitivity of the ATP assay to  $<10^4$  CFU/ml.

**ATP estimation of microbial quality of fresh-cut melon pieces during refrigerated storage.** Populations of native aerobic bacteria and ATP concentrations in fresh-cut melon prepared from chlorine-sanitized cantaloupe and honeydew and stored at 5, 10, and 15°C for up to 15 days are shown in Figures 2 and 3. Bacterial populations on fresh-cut cantaloupe pieces increased from an initial value of 1.61 to 7.95, 8.41, and 9.55 log CFU/g at 5, 10, and 15°C, respectively, over the 15-day storage period (Fig. 2). During the same interval, the ATP concentrations increased from 3.18 to 7.14, 7.88, and 8.89 log fg/g at 5, 10, and 15°C, respectively. Storage of fresh-cut pieces at 23°C was stopped at day 5 due to high microbial populations, including yeasts and molds; microbial populations and ATP concentrations in these fresh-cut samples were higher than those in samples stored at 5, 10, or 15°C (data not shown). For fresh-cut honeydew, both bacterial populations and ATP concentrations increased during storage but to lower final values than those for cantaloupe (Fig. 3). Similar results were obtained from fresh-cut melons prepared from whole melon sanitized with hydrogen peroxide (data not shown).

Total aerobic plate counts and ATP concentrations obtained during storage of fresh-cut cantaloupe and honeydew were highly positively correlated during storage at 5°C (Table 2). These data indicate that the ATP assay can be effectively used to determine the microbial quality of minimally processed fresh-cut melons during refrigerated storage. However, further studies are needed to determine the effect of geography, maturity, season of harvest, and storage conditions on the native microflora, especially yeasts and molds, and the associated concentrations of detectable ATP. Differences in the regression coefficient (*r*) between cantaloupe and honeydew and between fresh-cut pieces pre-

pared from sanitized versus unsanitized melons indicate the need to customize regression equations for prediction of microbial load for each melon type and fresh-cut process used. Differences in the y-intercept (*a*) are related to the initial microbial load, and differences in the slope (*b*) appear to be characteristic of the melon and sample type, regardless of microbial load. The bioluminescence ATP assay cannot distinguish ATP concentrations from individual classes or types of microflora, such as human bacterial pathogens, on the surface of fresh-cut cantaloupe and honeydew and thus cannot directly be used as an indicator of the safety of a product. However, the assay does provide an indication of total microbial load, which reflects the effectiveness of sanitization interventions applied to the whole melon and sanitary conditions during the cutting and packaging stages. Because of the versatility of the ATP assay, it can be utilized with modifications to monitor microbial load and/or sanitary conditions during preparation of fresh-cut melon and for determining microbial quality of fresh-cut melons during refrigerated storage.

This ATP bioluminescence assay might be useful to both processors and retailers for assessing the microbial quality of fresh-cut cantaloupe and honeydew melons. After establishment of baseline ATP concentrations for fresh-cut pieces, an unexpected spike in such values may indicate improper sanitizer preparation and/or use.

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